ACCURATE AND RELIABLE MULTI CHAMBER PCR CHIP WITH SAMPLE LOADING AND PRIMER MIXING USING VACCUM JACKETS FOR n × m QUANTITATIVE ANALYSIS

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ABSTRACT

This paper reports a high-throughput n × m well array PCR chip with an accurate and reliable method for sample loading and primers mixing. PCR mixture could be precisely introduced into each reaction chamber, which was pre-spotted and dried up with each primer set, by sucking the air through PDMS gas permeable wall. In this approach, the mixing of primer and sample at constant ratio was achieved and an equal amplification rate at an unique condition was ensured across the reaction chambers. Our method can be used to develop multi-target sample amplification chips for diagnostic applications.

KEYWORDS: PDMS gas permeability, high-throughput PCR chip, bubble elimination, sample evaporation

INTRODUCTION

Polymerase chain reaction (PCR) has become the ubiquitous method for nucleic-acid amplification and it has developed into an indispensable tool in clinical diagnostics. But, for clinical investigations, high throughput gene expression analysis is becoming inevitable. However, conventional technique is insufficient for multi-target-amplification because of their intrinsic single reaction design along with limiting factors like reagent and time consumptions. It is, therefore, necessary to develop a new device to incorporate parallelism and throughput on a single platform.

Until today, considerable efforts have been made to develop microfluidic PCR- chips with varying designs and materials for high-throughput, effective and faster DNA amplification. Glass or silicon substrates were generally used for high-throughput PCR chip fabrication[1, 2, 6]. In some cases, the chip has the reaction wells minimized, which either involves immobilization of primer pairs in a reaction well, or requires an expensive liquid-handling instrument [1, 6].

Despite promising results, significant challenges still remain, such as sample loading process and sample lost during DNA amplification at high temperature. Till now, few researchers have used microfluidics simply for loading samples into the chips and to control the sample lost. Quake and co-workers demonstrated microfluidic platform for distribution of 2μl PCR mixture to 400 independent reactors using 2860 integrated hydraulic valves and pneumatic pumps [3]. Kong et al [4] designed microfluidic chip for distributing 500 nl of PCR mixtures to each of four reaction chambers using mechanical valve array for sealing the wells. Recently, Ramalingam *et al* reported a capillary-driven microfluidics for loading PCR cocktail into reaction chambers and used semi-cured liquid PDMS prepolymer to prevent sample loss during PCR thermal cycling [2].

In our work, we exploited the gas-permeability of PDMS both for sample loading and for eliminating trapped-air from our chip. By this way, a constant amount and ratio mixing of primers and PCR mixture was achieved, which helped to get the same amplification rate in every reaction chamber in chip under unique condition. This method is applicable for n × m well array quantitative PCR analysis. We showed successful simultaneous amplification of three different target genes on our platform including the human β-Actin, the human sex-determining region Y (SRY), and the human Rhesus D (RhD). The fluorescence microscopy (Leica) was used to confirm the amplification of the target gene in each reaction chamber. Our approach resulted in a promising device capable of high-throughput DNA amplification at low-cost which is suitable for point-of-care clinical diagnosis even by non-specialist users.

EXPERIMENTAL

Device fabrication

The device contained three layers - the valve control layer on the top, the flow layer at the bottom, and the thin hybrid Parylene C-PDMS membrane sandwiched between them for valves as shown in Fig 1. Chip fabrication was described in our previous report [5]. Flow layer contained n arrays of m circular reaction chambers, each 800 μm in diameter and 200 μm in height, accommodating approximately 100 nl of PCR solution.

PCR device operation

Different primer sets were spotted on the respective reaction chambers on the flow layer and dried up at room temperature before attaching the control layer to it by oxygen plasma treatment. The valve control and vacuum suction ports of the chip were connected to two pressure-controlled outlets of the system for loading samples into the chip. During loading process, air was sucked through the vacuum suction port, the PCR mixture then loaded into the reaction chambers as air in the reaction chambers penetrated into air jacket through the permeable PDMS walls. After all reaction chambers were filled up with PCR mixtures without any air being trapped in the reaction chambers, all the valves were closed to ensure the complete isolation among the chambers and to avoid sample leakage and cross contamination among the chambers.

PCR amplification

A 295-bp segment of the human β-Actin, a 137-bp segment of SRY, and a 74-bp segment of RhD were amplified on the chip. The sequence of primers specific to above target genes are shown in Table 1.

Table 1. The nucleotide sequences of the primers

Gene	Forward primer	Reverse primer
β-actin gene	5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3'	5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3'
SRY gene	5'-TGG CGA TTA AGT CAA ATT CGC- 3'	5'-CCC CCT AGT ACC CTG ACA ATG TAT T-3'
RhD gene	5'-CCT CTC ACT GTT GCC TGC ATT-3'	5'-AGT GCC TGC GCG AAC ATT-3'

DNA amplification in chip was performed on the flat surface thermocycler (ASTEC). The thermal cycling program was commenced by heating at 95°C for 10 minutes to activate the polymerase and denature the initial DNA, followed by thermal conditions consisted of denaturing at 95°C for 15 sec, and annealing and extension at 65°C for 1 minute. Upon completion of up to 30 thermal cycles, the chip was kept at 25°C before fluorescence intensity measurement. The negative control experiment was conducted by replacing the template genomic DNA with nuclease-free water.

Fluorescence detection and analysis

Fluorescence microscope (Leica) with a ×10 objective was used to monitor fluorescence generated from reaction chamber after amplification. Mercury vapor lamp with filter was used as the excitation source. The fluorescence images were captured using a digital CCD camera. The difference in fluorescence intensities were used to distinguish between the positive and the negative control.

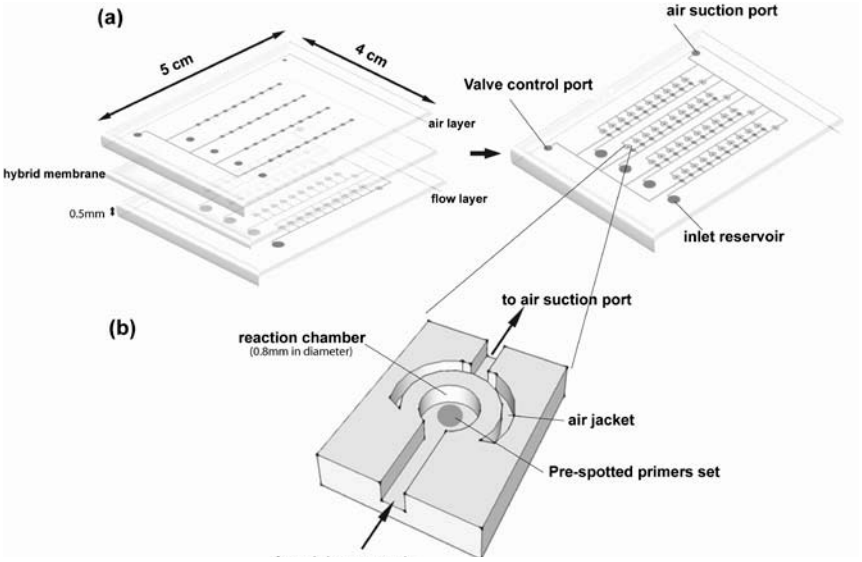

Figure 1. (a) The multi-chamber PCR chip platform; (b) The magnification of the structure of PCR reaction chamber

RESULTS & DISCUSSION

Sample loading in chip

In this experiment, the positive pressure and the negative pressure were set at 480 hPa and -400 hPa, respectively for the sample loading into the chip. The reaction chambers were gradually filled with sample while the air was being sucked through the air jacket as shown in Fig 2. The entire reaction chamber was filled with the methyl green solution and no trace of air bubbles was observed after completion of the loading process.

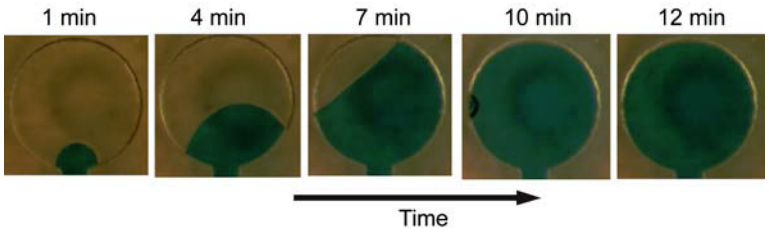

Figure 2. Sample loading in PCR chip under evacuation process over time.

Bubble elimination and evaporation

There are three main causes of the sample loss during PCR - the trapped air in the reaction chamber from sample loading, the dissolved gas in both PCR mixture solution and PDMS, and the evaporation of water from PCR mixture through PDMS at high temperature during thermal cycles. The first two causes were eliminated along with sample loading by evacuation as shown in Fig.2. Air was completely removed during the sample loading process by evacuation. The sample evaporation issue was taken care of by adding glycerol into the PCR mixtures which helped to increase the boiling point of the PCR mixture and thereby inhibit the sample evaporation [5]. From Fig 3, it could be concluded that the addition of glycerol up to 20% in weight into PCR mixture did not affect DNA amplification process and the Ct value remained unchanged upon the addition of glycerol into the PCR mixture.

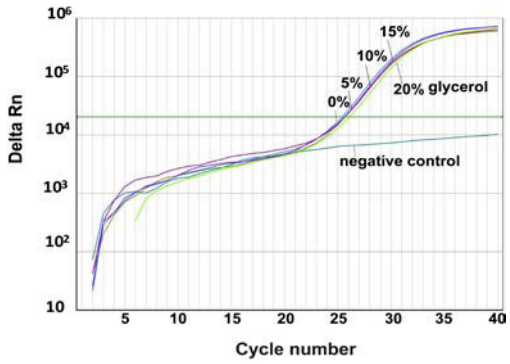

Figure 3. Real time PCR of β-Actin gene with varying concentration of glycerol

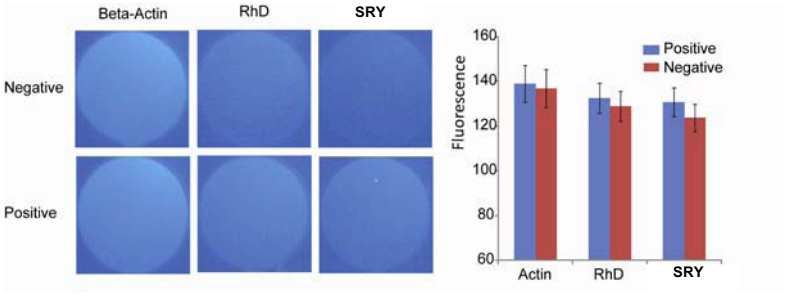

Figure 4. Fluorescence intensity of positive samples and negative samples in chip after 30 thermal cycles of PCR

Three different target gene sequences of human template DNA related to β-Actin, RhD and SRY were amplified in our multi-array PCR chip. 1 µl of each primers pair (3 µM for each) were pre-loaded and dried in different reaction chambers. The reaction mixtures for on-chip PCR consisted of 10× GeneAmp PCR buffer II , 0.2 mM each dNTP, 3.5 µM $MgCl_2$, 0.125 U/µl of AmpliTaq Gold DNA polymerase, 0.2 ng/µl of human template DNA; 1× SYBR green 1, and 20% (w/w) glycerol. The PCR mixture was loaded into the reaction chambers and DNA amplification was performed right after. During PCR process, the valves were kept closed firmly to avoid sample leakage and cross contamination among the reaction chambers.

Fig 4 shows the fluorescence image of each reaction chamber after simultaneous amplification of three different target DNAs on a single chip. In presence of the target DNA, a high fluorescence signal was obtained from that reaction chamber. The fluorescence intensity clearly distinguished between positive and negative chambers. The different fluorescence intensities of different primers are to be considered individually in each case and it should be compared between the chambers containing the same type of primers.

CONCLUSION

In this paper, a novel method for loading sample into PDMS high-throughput PCR chip with the reaction chamber volume in nanolitre range has been developed. Gas permeability of PMDS was exploited successfully for sample introduction. We achieved the simplified multi-array-structure for PCR with a constant amount/ratio mixing of primers and PCR mixture without integrating any complicated component onto the chip. The microchamber array chip described in this report can be used to amplify multiple DNA targets at trace amounts in nanoliter volume. This device is a promising candidate for multi-target nucleic acid analysis device due to its low-cost and high- throughput detection abilities, which can be applied for point-of-care clinical diagnosis.

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